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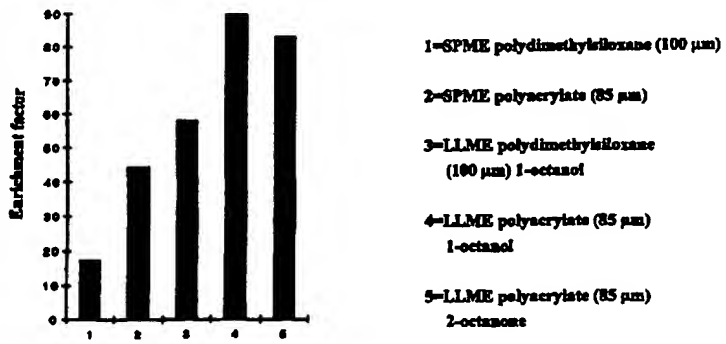
(54) Title: METHOD FOR LIQUID-LIQUID MICROEXTRACTION

SPME versus LLME
in aqueous buffer
5 nmol/ml of diazepam in 1 M acetate buffer pH 5,5



(57) Abstract

A method of extraction and analysis of high boiling and/or macromolecular substances in complex materials, particularly biological material, comprising the following sequence of steps: (a) to provide a suitable carrier; (b) possibly to modify the carrier surface; (c) to immobilize a solvent on the surface of the carrier; (d) to contact the surface modified carrier with the material, possibly in the form of a solution, containing the substance to be analyzed; (e) to concentrate and fixate the substance to be analyzed in the solvent fixed on the carrier; and (f) to bring the carrier with the concentrated substance to be analyzed into an analytical apparatus for desorption and analysis. As carriers there are preferably used fibres having a diameter of 50 to 300 µm, most preferably 100 to 200 µm, and of a length of about 2 cm.

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Method for Liquid-Liquid Microextraction

The present invention relates to a method for liquid-liquid microextraction, more specifically, a method for the extraction and analysis of high boiling and/or macromolecular substances in complex materials, particularly biological materials.

Sample preparation is the most tedious and time consuming step in analysis of drugs present in the pg/ml to µg/ml concentration range in biological liquids such as blood, serum, plasma or urine. The principal steps of sample preparation involves concentration of the analytes and removal of as many interfering compounds as possible. The use of an extraction technique is common in the pre-treatment of most types of samples.

Liquid-liquid extraction or LLE is one of the most frequently used techniques. In liquid-liquid extraction the analyte is extracted from an aqueous matrix such as serum, plasma or urine into a water immiscible low boiling solvent. The type of matrix and the solvent are optimized to enable selective extraction of the analytes. The solvent is evaporated and the analytes reconstituted in a sufficiently small amount of solvent to obtain a concentrated solution for injection into an analytical instrument such as a gas chromatograph (GC) or a liquid chromatograph (HPLC). The advantage of LLE is the wide variety of solvents and solvent mixtures available to achieve a selective extraction of the analytes. However, LLE have certain disadvantages, such as the time required, the relatively large sample volume required (a minimum of 0.5-1 ml), the use of a large volume of organic solvents which may be toxic, the formation of emulsions, and the need to concentrate the extract by evaporation of solvent.

Solid Phase Extraction or SPE avoids or minimizes the disadvantages of LLE listed above. The time required is

reduced, the technique permits small sample volumes, it requires small volumes of solvents, and the formation of emulsions is avoided. If necessary, a low boiling solvent can be used in the final elution, and the solvent may be evaporated and the analytes reconstituted in a small volume of solvent before injection into an analytical instrument such as a GC or an HPLC.

SPE can easily be automated in off-line or on-line systems. Certain of these carry out all the SPE process steps automatically, but the transfer of the eluates from SPE to a GC or an HPLC is manual. Others are able to transfer automatically the eluates to an HPLC injector to enable fully automated and unattended sample preparation and HPLC.

Fully automated on-line sample preparation and analysis was until recently only possible when the final separation method was HPLC. The ease of combining automated sample preparation with HPLC separation was one of the reasons why HPLC has developed into the major analytical separation method for drugs in biofluids. However, GC with capillary columns has a larger separation power than HPLC, and many specific and highly sensitive detectors are available. The development of new methods for sample preparation and injection techniques with a potential for on-line sample preparation and GC analysis has been given a great deal of attention lately.

A promising experimental configuration recently introduced is the so-called solid phase microextraction or SPME, in which the solid phase is a fibre of various materials such as uncoated, fused silica or optionally such silica coated with polydimethylsiloxane; polyimide; liquid crystal polyacrylate; Carbowax or graphite. These materials are all non-volatile and act as solid sorbents for partitioning of the analytes. For protection the fibre is mounted in the needle of a micro syringe. Upon sampling, the micro syringe plunger is depressed to lower the fibre in the liquid sample for a

specific period of time. The analytes are adsorbed on the fibre aided by agitation of the sample. After adsorption of the analytes, the fibre is withdrawn into the needle. The analytes are then thermally desorbed by introducing the needle into the heated injection port of a gas chromatograph. The procedure can also be applied to the vapour phase in equilibrium with a liquid or solid to obtain head space sampling of volatiles. SPME can easily be automated by commercially available auto-injectors for GC. SPME can also be used in combination with HPLC as separation method.

With respect to the technology in this field, special reference shall be made to NO 923826, filed October 1, 1992, in the name of Janusz B. Pawliszyn, and to later literature by the same author.

SPME was developed for the analysis of organic compounds in water samples. When SPME was applied to bioanalytical samples several difficulties were observed. The method showed insufficient enrichment in most cases. Drugs are high boiling compounds with a molecular weight of from 200 to 400 and are sorbed slowly onto the fibre. The enrichment will therefore take a long time and may require as much as 10 to 15 minutes. The biological sample solution has a high viscosity as compared with a water sample and the sorption of drugs onto the fibre is greatly reduced in a viscous sample. The selectivity was poor because the fibres for SPME are non-selective in nature. The consequence of a non-selective enrichment is many interfering compounds in the chromatograms. Biological macromolecules, such as proteins, are sorbed onto the fibre, and the fibre was rapidly contaminated.

The object of the present invention is to reduce the disadvantages of the SPME technology and to provide a process that enhances the possibility of analyzing high boiling substances in complex samples, for example biological

material and even environmental samples, and at the same time to increase the selectivity without renouncing the advantages of prior art.

Any suitable material may in principle be used as carrier, but the invention will be described with a fibre as carrier.

According to this, the present invention relates to a process of extraction and analysis of high boiling and/or macromolecular substances in complex materials, particularly biological materials, and this process is characterized by the following sequence of steps:

(a) to provide a suitable carrier,
(b) possibly to modify the carrier surface,
(c) to immobilize a solvent on the surface of the carrier,
(d) to contact the surface modified carrier with the material, possibly in the form of a solution, containing the substance to be analyzed,
(e) to concentrate and fixate the substance to be analyzed, in the solvent fixed on the carrier, and
(f) to bring the carrier with the concentrated substance to be analyzed into an analytical apparatus for desorption and analysis.

Preferably a fibre is used as carrier.

The liquid-liquid microextraction, or LLME, of the invention reduces or minimizes the problems encountered with SPME. The invention combines the advantages of LLE with the advantages of SPME.

In contrast to LLE, LLME is based on extraction onto an immobilized solvent. A carrier is used to immobilize the solv nt. The amount of solvent which is immobilized is in the magnitude range of 1-5 µl. The carrier with the

immobilized solvent is inserted into the sample solution. The chemical properties of the biological matrix are altered, for example by changing the pH, in order to favour partitioning of the analytes into the immobilized solvent. Upon agitation the partitioning is generally complete within 2-6 minutes. The carrier with the enriched analytes dissolved in the immobilized solvent is then transferred to the injection port of an analytical instrument such as a GC which is maintained at a sufficient injection temperature. The immobilized solvent and the analytes evaporate and are separated on the GC column. No evaporation of solvent is necessary prior to injection. The total amount of solvent used for extraction is injected into the chromatograph and no solvent is wasted. In LLME only 1-5 µl of solvent is used for analysis, a circumstance which greatly reduces the risk of solvent exposure to operators and to the environment.

Solvents suitable for LLME can be divided into high boiling solvents and low boiling solvents. Solvents having a boiling point in the range of 100 - 200°C are classified as high boiling solvents. These solvents evaporate slowly at room temperature and are well suited for LLME. These solvents have also powerful concentrating effect in splitless injection in capillary GC. A high starting temperature in temperature programmed GC can be used, and a gain in analysis time is obtained. Examples of high boiling solvents for LLME are alcohols such as octanol, diols such as 1.2-butanediol, ketones such as 22-octanone, ethers such as dibutylether, and esters such as pentylacetate. Low boiling solvents such as those ordinarily used in LLE can also be used in LLME. These solvents can easily be evaporated from the carrier after enrichment, leaving high boiling analytes in a solvent free environment on the carrier. A solvent free injection can thereby be performed. Organic solvents have a low viscosity as compared with the sorbents used in SPME. High molecular weight substances such as drugs partition rapidly into the immobilized solvent as compared with the

high viscous SPME coating, resulting in short sorption time as compared with SPME. The selectivity can be optimized by varying of the chemical type of solvent and by mixing solvents. Macromolecules such as proteins are not soluble in organic solvents. This gives less contamination of the carrier, and the life time of the carrier is greatly extended as compared with the SPME fibre. LLME can in particular be used to analyze drugs where the sample volume is less than 1 ml.

The requirements to be fulfilled by a carrier used in LLME is that it must provide the necessary surface area for immobilizing the solvent so as thereby to achieve rapid extraction of the analytes from the aqueous biofluid. In addition, it must be able to immobilize 1-5 µl of solvent, be solvent resistant, be heat resistant up to 300°C and be pH insensitive, since it is generally used for extraction in the pH range of 2 to 12.

Many materials can act as carriers to immobilize solvents. Examples of such materials are porous polymers; paper, porous glass, silica or metal; glass, silica or metal needles; glass, silica or metal spirals or gauzes. The surface can be modified to enhance immobilization of organic solvents having hydrophobic bindings (Van der Waal's bindings) and deactivated to prevent strong interaction with analytes having polar bindings (dipole interaction and hydrogen bonding) or ionic interaction which may lead to reduced evaporation of the analyte from the carrier. In addition, the carrier should be chemically inert to prevent catalytic degradation of the analytes during desorption in the heated GC injector.

Various devices can be used to bring the carrier into an analytical instrument, for example a GC. During the period from 1960 to 1980 a number of apparatuses were constructed for the introduction of solid samples into a GC. Some of

these devices were based on modified syringes having a needle inside of a syringe ne dle. The advantage of the modified syringe design is that it can penetrate the septum of a GC injector. Other devices were based on special holders which were screwed on the injection port of the GC. A carrier for a solid sample was attached to a rod inside of the holder. By a pressure on the rod, the carrier for the solid sample was inserted into the heated injector of the chromatograph, and the solid sample evaporated. In principle, both modified syringes and modified injection devices for solid samples can be used for LLME. Modified syringes, like those used in SPME, offer the advantage that existing devices can be used also for LLME. However, instead of using a fibre coated with a SPME sorbent, a fibre for LLME is used.

LLME can also be used as a sampling technique for HPLC when corresponding injectors are used and when the HPLC mobile phase is compatible with the immobilized solvent used in LLME.

Analysis of a liquid sample through the metod of LLME and by means of a modified syringe involves the following steps:

First, the fibre is withdrawn into the needle of the syringe, and the needle is used to penetrate septum of a solvent vial. The fibre is lowered into the solvent by depressing the plunger. The solvent is immobilized onto the fibre and the plunger is withdrawn so that the fibre is withdrawn into the needle before the needle then is withdrawn from the solvent vial.

Next, the needle is used to penetrate the sample vial, and the fibre is lowered into the sample by depressing the plunger. Analytes are partitioned onto the immobilized solvent upon agitation of the sample vial. The plunger is then withdrawn so that the fibre is withdrawn into the needle before this is removed from the sample solution.

Finally, the solvent and the enriched analytes are evaporated in the injector of a gas chromatograph followed by separation on the GC column.

Most drugs used in human medicine are basic drugs, and analysis of basic drugs has been shown here in order to demonstrate the advantages of LLME in relation to SPME. In standard procedures these drugs are extracted from 0.5-1 ml of plasma by LLE or SPE. The extract is evaporated and the drugs are reconstituted in 50-100 µl of solvent. 1-2 µl is injected into a GC for separation and analysis. The amount of drug injected into the chromatograph by this procedure is equivalent to the introduction of 10-20 µl of plasma. In an equivalent enrichment based on LLME or SPME an amount of drug present in 10-20 µl of plasma will be quantitatively enriched on the fibre. If the enrichment is lower than in a standard procedure, microextraction will not provide the necessary enrichment for bioanalysis of drugs.

The invention will be further illustrated in the following examples with reference to the accompanying drawings where:

- Figure 1 illustrates the immobilization of solvent;
- Figure 2 shows enrichment based on the use of SPME, respectively LLME;
- Figure 3 compares SPME with LLME in analysis of diazepam in human plasma;
- Figure 4A and 4B shows the chromatograms for a blank plasma sample and a plasma sample having a different analyte;
- Figure 5A and 5B shows the chromatograms for a blank plasma sample and plasma samples having different analytes; and
- Figure 6A and 6B shows the chromatograms for a blank plasma sample and a plasma sample having an additional analyte.

Preliminary experiments:

The principle of LLME can be demonstrated by the following experiment:

A cleaned filter paper having the dimensions of 1 mm x 5 mm was used as carrier for immobilization of 5 to 8 µl of chloroform. The filter paper with immobilized chloroform is inserted into a 1 mmol/ml aqueous solution of diazepam at pH 5.5 and agitated for 1 minute. The solvent is partly evaporated before the filter paper is injected into the injector port of a GC with a solid injector screwed onto the injection port as described above. Analysis was carried out on a packed GC column and an FID was used for detection. The experiment showed an enrichment factor for diazepam from the aqueous solution of 60 to 80.

Example 1

In this example the benzodiazepines diazepam and its metabolite N-desmethyldiazepam were determined in human plasma. Diazepam is highly bound to proteins in human plasma (98%) and in total drug analysis measures have to be taken to reduce the protein binding prior to the enrichment by LLME and SPME. For the analysis of the highly protein bound basic drugs in plasma, such as benzodiazepines, a general sample pretreatment procedure has been developed.

To an amount of 450 µl of plasma there was added 50 µl methanol containing prazepam as internal standard. The mixture was agitated for 1 minute. The addition of methanol to plasma reduces the protein binding of the benzodiazepines. Thereafter, plasma proteins were precipitated by addition of 100 µl 1 M trichloroacetic acid solution. After centrifugation at 13,000 rpm for 10 minutes, 100 µl 5 M acetate buffer pH 7 was added to the 400 µl supernatant, thus ensuring a

final pH in the plasma supernatant of 5.5. Diazepam has its highest partition coefficient both into an immobilized organic solvent and into the SPME stationary phase at a pH equal to 5.5.

A polyacrylate SPME fibre was used as carrier for solvents in LLME. Both SPME analysis and LLME analysis of the plasma supernatant were carried out to demonstrate the differences between the two techniques. LLME was carried out with octanol as solvent. Octanol was immobilized on the SPME fibre by inserting the fibre into an octanol solution for 2 minutes. The procedures for LLME and SPME with respect to plasma supernatant are identical. The fibres were inserted into the plasma supernatant. Partitioning onto the fibres was carried out by agitation of the sample vial for 4 minutes. The fibres were thereafter injected into the splitless injector of a capillary GC at 300°C. Desorption of the analytes was complete after 1 minute. The analytes were separated on a capillary column and detected by an NPD detector.

The volume of immobilized solvent is an essential parameter in LLME and is dependant upon the type of solvent, the coating on the carrier, the surface area and the surface tension on the carrier. The fibres designed for SPME were investigated as LLME carriers. The volume of solvent immobilized on various SPME fibres is shown in Figure 1. The polyacrylate coating was the best on the commercially available SPME fibres and was capable of immobilizing 1.5 µl of octanol and 3 µl of 2-octanone. A number of high-boiling organic solvents were investigated, but all resulted in insufficient immobilization and/or incompatibility with the coating on the SPME fibres.

The enrichment of diazepam in an aqueous acetate buffer pH 5.5 was determined by SPME and LLME for comparison of the two techniques. The enrichment factor was defined as the

equivalent volume in µl of samples which were enriched, i.e., with an enrichment factor of 10 the amount of analyte in 10 µl of sample was concentrated on the coating or the immobilized solvent. By LLME with a polyacrylate coating an enrichment factor of about 90 was obtained with both octanol and 2-octanone as immobilized solvents. This clearly demonstrates the difference in selectivity between octanol and 2-octanone towards diazepam, since twice the amount of 2-octanone was required for enriching the same amount on the SPME fibre. The enrichment obtained by SPME was about 40 as shown in Figure 2.

The enrichment of diazepam from plasma was less efficient since the biological sample contained several endogenous compounds and was highly viscous compared with the acetate buffer as shown in Figure 3. A decrease in partitioning was observed in both LLME and SPME. An enrichment factor of about 20 was obtained with LLME (octanol) while SPME for the same sample gave insufficient enrichment for bioanalysis of diazepam, with an enrichment factor of 6 with a sorption time of about 6 minutes.

The LLME procedure for the determination of diazepam in human plasma was validated and found to be reproducible. The standard curves (calibration graphs) were found to be linear in the concentration range 0.25-7.5 nmol/ml with correlation coefficients of 0.9994 or better. The intra- and interassay validation of the procedure is shown in Table I. The limit of detection at a signal-to-noise ratio of 3 (S/N=3) was found to be 0.10 nmol/mo and in agreement with other procedures for the determination of diazepam in human plasma. Chromatograms from the LLME analysis of benzodiazepines are shown in figure 4.

Table I

Intra- and interanalysis variations according to LLME, expressed as an average of parallel assays ± standard deviation (S.D.) and relative standard deviation (R.S.D.).

Added Concentration (nmol/ml)		Measured Concentration ± S.D.	R.S.D.
Intra-analysis			
(n=6)	0.50	0.48 ± 0.02	4.2 %
	3.00	2.92 ± 0.17	5.8 %
	5.00	4.96 ± 0.16	3.2 %
Inter-analysis			
(n=6)	0.50	0.48 ± 0.03	6.3 %
	3.00	2.94 ± 0.19	6.5 %
	5.00	4.98 ± 0.28	5.6 %

Example 2

The potential of LLME in bioanalysis of drugs was investigated by analysis of opiates, viz., codeine, ethyl morphine, morphine, 6-monoacetylmorphine and pholcodine and by the analysis of a tricyclic antidepressant drug, viz., clozapine, all in human plasma. The plasma samples were pre-treated in the same manner as for the analysis of benzodiazepines: addition of methanol and internal standard to ensure reduction of protein binding prior to protein precipitation. LLME was carried out with octanol as solvent, and partitioning of the analytes into the solvent was carried out by agitation of the sample vial for 4 minutes. The immobilized octanol and the analyte were desorbed in the injection port (300°C) of a GC, and the separation was carried out with minor modifications of the temperature program used for the separation of the benzodiazepines and detected by NPD. The LLME analysis of the opiates as well as the analysis of the

antidepressant were successful. The chromatograms from the LLME analysis of opiates and clozapine in human plasma are shown in Figures 5 and 6.

LLME offers several advantages compared with SPME, viz:

- rapid sorption time
- selectivity
- high enrichment
- less contamination of the carrier/fibre
- reduced adsorption of macromolecules.

LLME provides short sorption times compared with SPME as high molecular weight substances such as drugs partition rapidly into the immobilized solvent as compared with the high viscous SPME coating. The selectivity of LLME can be optimized for each specific application by variation of the chemical nature of the solvent and by mixing of solvents, while SPME is confined to the limited number of commercially available coatings. The number of organic solvents and mixtures of solvents available for LLME will always be greater than the number of coatings for SPME. The enrichment obtained by LLME was satisfactory for the bioanalysis of drugs and enables the analysis of human plasma samples even though non-optimum SPME fibres were used as liquid carriers. SPME did not provide sufficient enrichment for bioanalysis of most drugs. Macromolecules such as proteins are not soluble in organic solvents and this results in less contamination of the carrier and consequently increased life time of the carrier as compared with the SPME fibre. By optimizing LLME the organic solvent will cover the carrier completely, and because macromolecules will not adsorb to the carrier, a pretreatment of samples with macromolecules, for example plasma, can be carried out without protein precipitation, resulting in reduc d sample preparation time and manual sample treatment.

The potential for LLME as a n w technique for the d termination of drugs in bioanalysis and a supplementary technique to SPME has been demonstrated with non-optimum carriers.

Thus, LLME quite clearly offers sufficient enrichment for bioanalysis of drugs, high selectivity and short sample preparation time. The applicability of the LLME technique of the invention has been shown for analysis of benzodiazepines, opiates and anti-depressants, but it is obvious that one does not depart from the spirit and framework of the invention if the technology of the invention should be used for other substances or if it should be based on other matrices.

Patent Claims

1.
A method of extraction and analysis of high boiling and/or macromolecular substances in complex materials, particularly biological material,
c h a r a c t e r i z e d b y the following sequence of steps:

(a) to provide a suitable carrier,
(b) possibly to modify the carrier surface,
(c) to immobilize a solvent on the surface of the carrier,
(d) to bring the carrier from step c) in contact with the material, possibly in the form of a solution, containing the substance to be analyzed,
(e) to concentrate and fixate the substance to be analyzed, in the solvent fixed on the carrier, and
(f) to bring the carrier with the concentrated substance to be analyzed into an analytical apparatus for desorption and analysis.

2.
A method according to claim 1,
c h a r a c t e r i z e d i n that the carrier employed is a fibre.

3.
A method according to claims 1 and 2,
c h a r a c t e r i z e d i n that the fibre has a diameter of 50 to 300 µm and a length of about 2 cm.

4.
A method according to any of the preceding claims,
c h a r a c t e r i z e d i n that the fibre has a diameter of 100 to 200 µm.

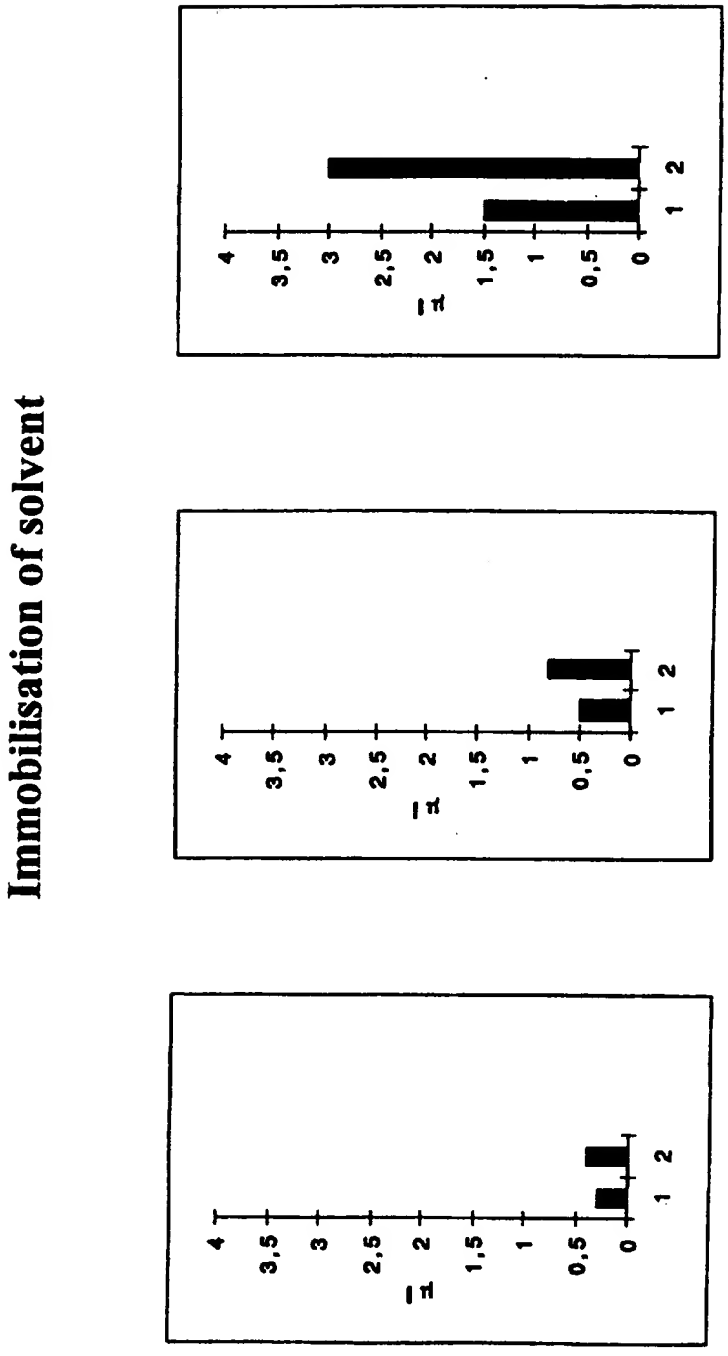


Fig. 1

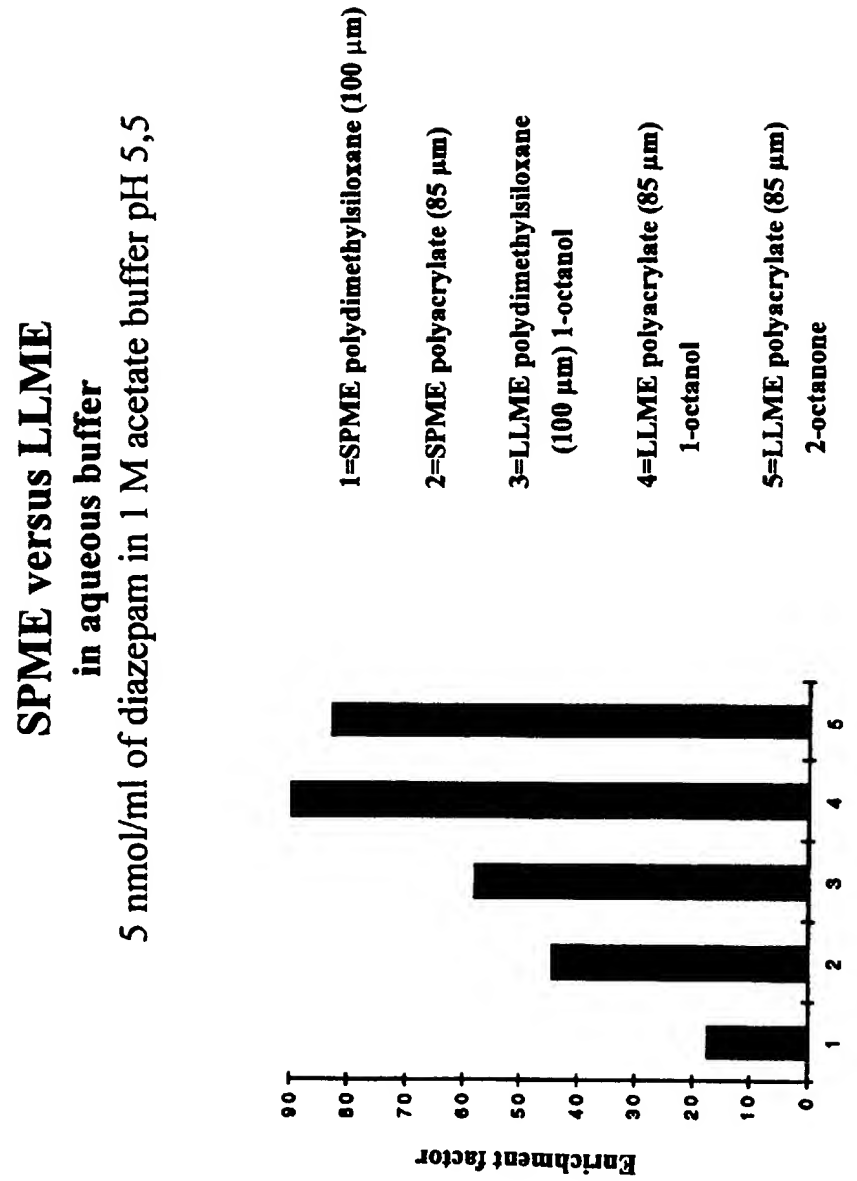
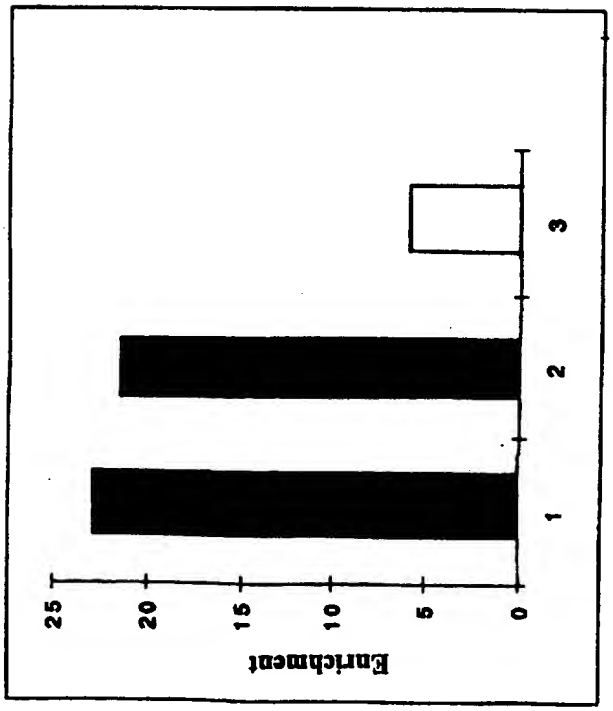


Fig. 2

Comparison SPME/LLME
analysis of diazepam in human plasma

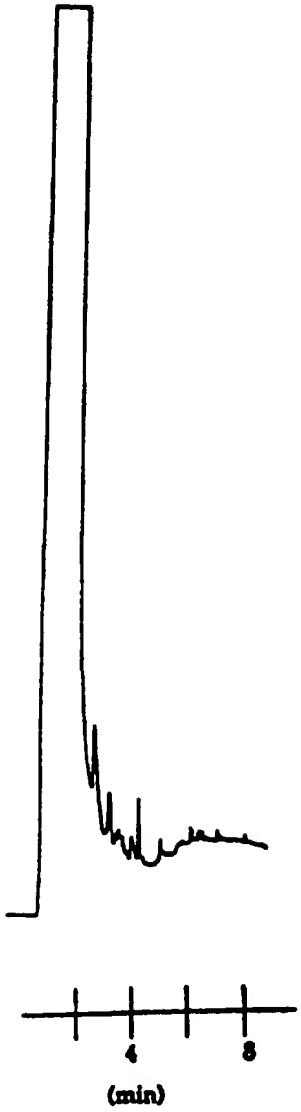


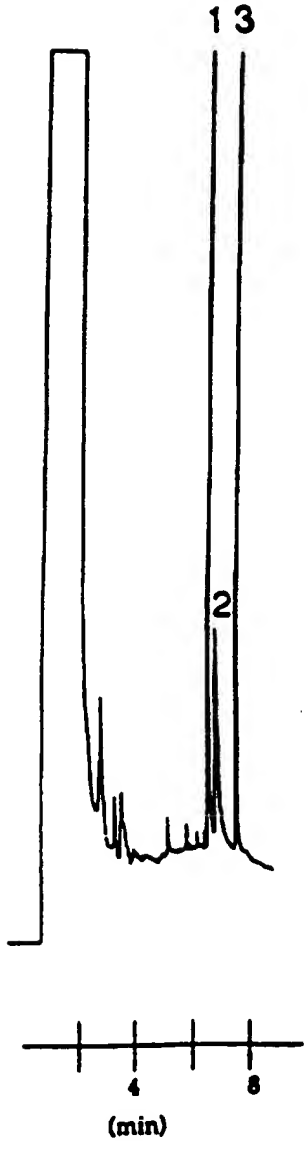
1=LLME of a plasma sample spiked after to precipitation (polyacrylate fibre)

2=LLME of a plasma sample spiked prior to precipitation (polyacrylate fibre)

3=SPME of a plasma sample spiked prior protein precipitation (polyacrylate fibre)

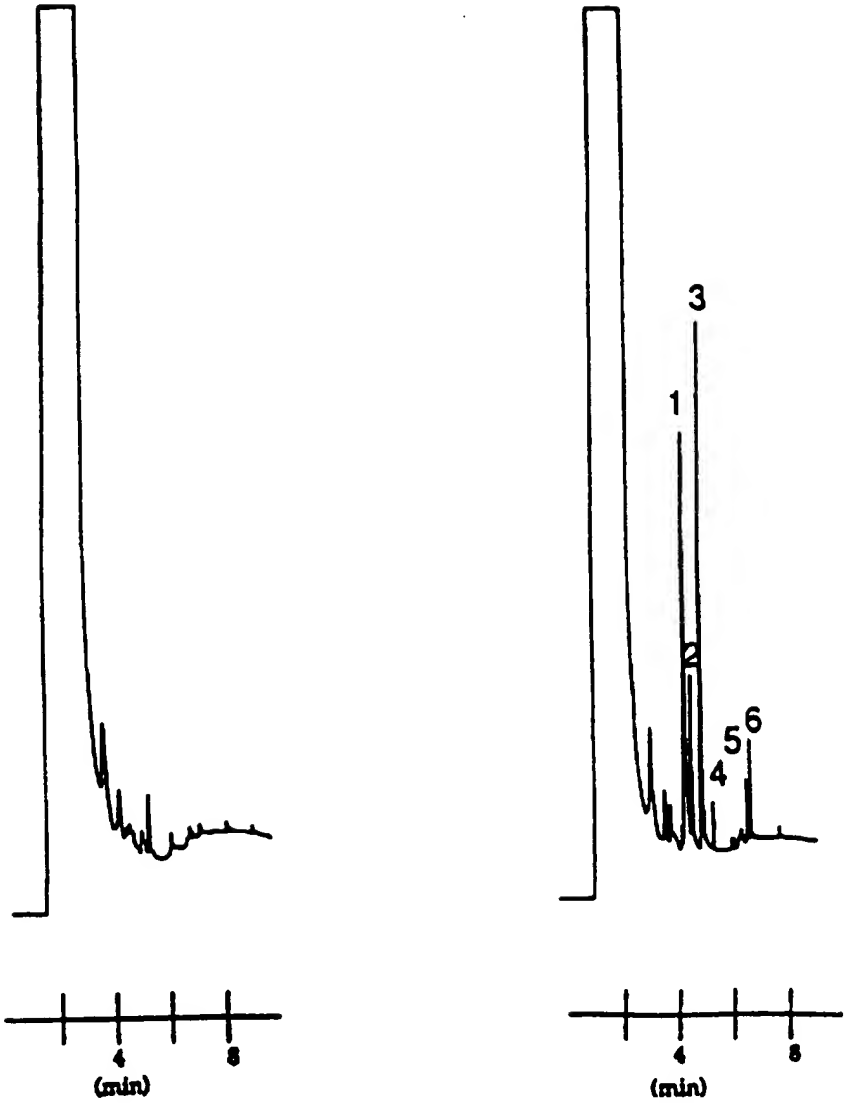
Fig. 3





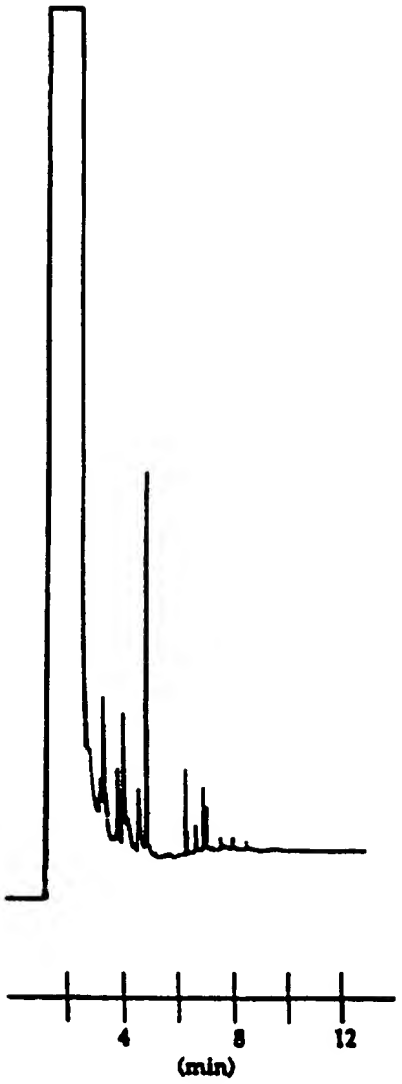
Chromatograms of (A) a blank plasma sample and (B) a plasma sample spiked with 5 nmol/ml of: 1=diazepam, 2=N-desmethyldiazepam and 3=prazepam (IS)

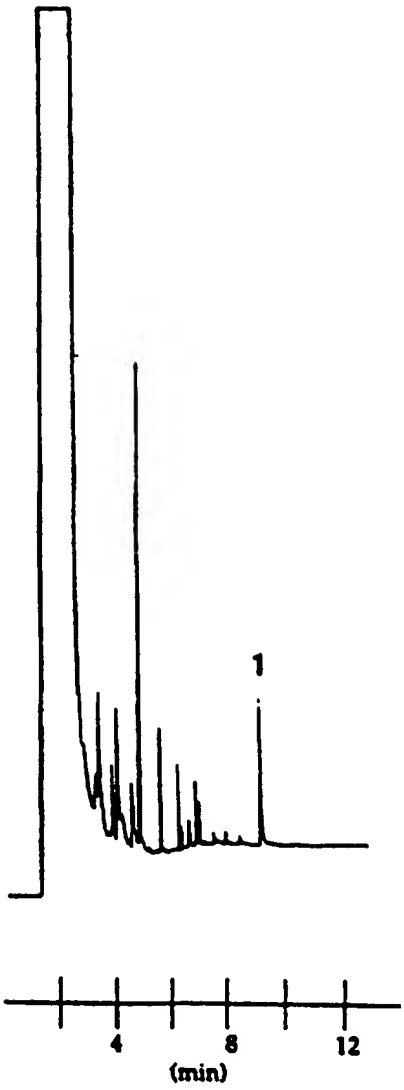
Fig. 4A Fig. 4B



Chromatograms of (A) a blank plasma sample and (B) a plasma sample spiked with 5 nmol/ml of: 1=codeine, 2=ethylmorphine, 3=morphine, 4=6-monoacetylmorphine (6-MAM), 5=nalorphine (IS) and 6=pholcodine

Fig. 5A Fig. 5B





Chromatograms of (A) a blank plasma sample and (B) a plasma sample spiked with 1 nmol/ml of clozapine

Fig. 6A

Fig. 6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NO 97/00010

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 1/40
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9115745 A1 (PAWLISZYN, JANUSZ, B.), 17 October 1991 (17.10.91), page 2, line 28 - page 3, line 19 --	1-4
A	ANALYTICAL CHEMISTRY, Volume 59, No 8, April 1987, Janusz Pawliszyn et al, "Sample Introduction for Capillary Gas Chromatography with Laser Desorption and Optical Fibers", page 1475 - page 1478, see page 1476, Experimental section --	1-4
A,E	EP 0730143 A2 (MALLINCKRODT BAKER, INC.), 4 Sept 1996 (04.09.96), page 3, line 3 - line 51 --	1-4

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 1997	26 -05- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Ulla Granlund Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/NO 97/00010

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2079337 A	03/10/91
		DE 69102700 D,T	12/01/95
		EP 0523092 A,B	20/01/93
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EP 0730143 A2	04/09/96	AU 4554496 A	05/09/96
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		US 5576217 A	19/11/96

Form PCT/ISA/210 (patent family annex) (July 1992)